Molecular Analysis of Radiation-Induced *albino* (*c*)-Locus Mutations That Cause Death at Preimplantation Stages of Development

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ABSTRACT

Deletion mutations at the *albino* (*c*) locus have been useful for continuing the development of fine-structure physical and functional maps of the *Fes–Hbb* region of mouse chromosome 7. This report describes the molecular analysis of a number of radiation-induced *c* deletions that, when homozygous, cause death of the embryo during preimplantation stages. The distal extent of these deletions defines a locus, *pid*, (*pre*implantation *d*evelopment) genetically associated with this phenotype. The proximal breakpoints of eight of these deletions were mapped with respect to the *Tyr* (tyrosinase; *albino*) gene as well as to anonymous loci within the *Fah–Tyr* region that are defined by the *Pmv-31* viral integration site and by chromosome-microdissection clones. Rearrangements corresponding to the proximal breakpoints of two of these deletions were detected by Southern blot analysis, and a size-altered restriction fragment carrying the breakpoint of one of them was cloned. A probe derived from this deletion fusion fragment defines a locus, *D7Rn6*, which maps within (or distal to) the *pid* region, and which discriminates among the distal extents of deletions eliciting the *pid* phenotype. Extension of physical maps from *D7Rn6* should provide access both to the *pid* region and to loci mapping distal to *pid* that are defined by *N*-ethyl-*N*-nitrosourea-induced lethal mutations.

OVER 100 mutations involving the *albino* (*c*) locus in mouse chromosome 7 have been recovered from the progeny of irradiated males and females at Oak Ridge National Laboratory and the MRC Radiobiology Unit at Harwell (RUSSELL, RUSSELL and KELLY 1979; GLUECKSOHN-WAELSCH 1979). These mutations are either viable, or prenatally or postnatally lethal, in the homozygous state (ERICKSON, GLUECKSOHN-WAELSCH and CORI 1968; LEWIS, TURCHIN and GLUECKSOHN-WAELSCH 1976; LEWIS 1978; LEWIS, TURCHIN and WOJTOWICZ 1978; RUSSELL, RUSSELL and KELLY 1979; RUSSELL and RAYMER 1979; GLUECKSOHN-WAELSCH 1979; RUSSELL, MONTGOMERY and RAYMER 1982). Genetic, phenotypic, and now molecular, analyses of the radiation-induced lethal mutations have suggested that they are deletions that remove neighboring genetic loci important for normal development (see RINCHIK and RUSSELL 1990, for review).

A number of prenatally lethal *c* deletions have been analyzed with respect to the developmental stage at which arrest of embryogenesis occurs. For example, it was recognized early that embryos homozygous for the c^{6H} deletion die around the time of gastrulation (LEWIS, TURCHIN and GLUECKSOHN-WAELSCH 1976), whereas c^{25H} homozygotes fail to elicit a decidual response and die before implantation at about 2.5 days (LEWIS 1978). In an analysis of a larger series of 26 independent, prenatally lethal *c* mutations induced at Oak Ridge, RUSSELL and RAYMER (1979) provided evidence for an additional 13 "implantation"-lethal and 13 "preimplantation"-lethal mutations. Pairwise complementation analyses among members of this larger series of lethal mutations allowed for the construction of a linear complementation/deletion map of a 6- to 11-cM chromosomal interval surrounding the *c* locus and provided evidence for a number of complementation groups of deletions (see Figure 1) (RUSSELL, RUSSELL and KELLY 1979; RUSSELL, MONTGOMERY and RAYMER 1982; see RINCHIK and RUSSELL 1990, for review).

More detailed histological analyses of four deletions that affect a *c*-distal region thought to be required for survival of the embryo after implantation (the original "Bi" complementation group of RUSSELL, MONTGOMERY and RAYMER 1982) have demonstrated that two different phenotypes are exhibited by deletion homozygotes (NISWANDER *et al.* 1988, 1989). One group of three deletions (c^{11DSD}, $c^{5FR60Hg}$ and c^{2YPSj}) fails to develop embryonic ectoderm and dies at about day 8.5, whereas another group of two deletions ($c^{4FR60Hd}$ and the c^{6H} deletion cited above) fails to develop extraembryonic ectoderm and dies a full day earlier. The proximal breakpoints of these deletions have been mapped with respect to cloned loci proximal to *c* (NISWANDER *et al.* 1991), and restriction fragments

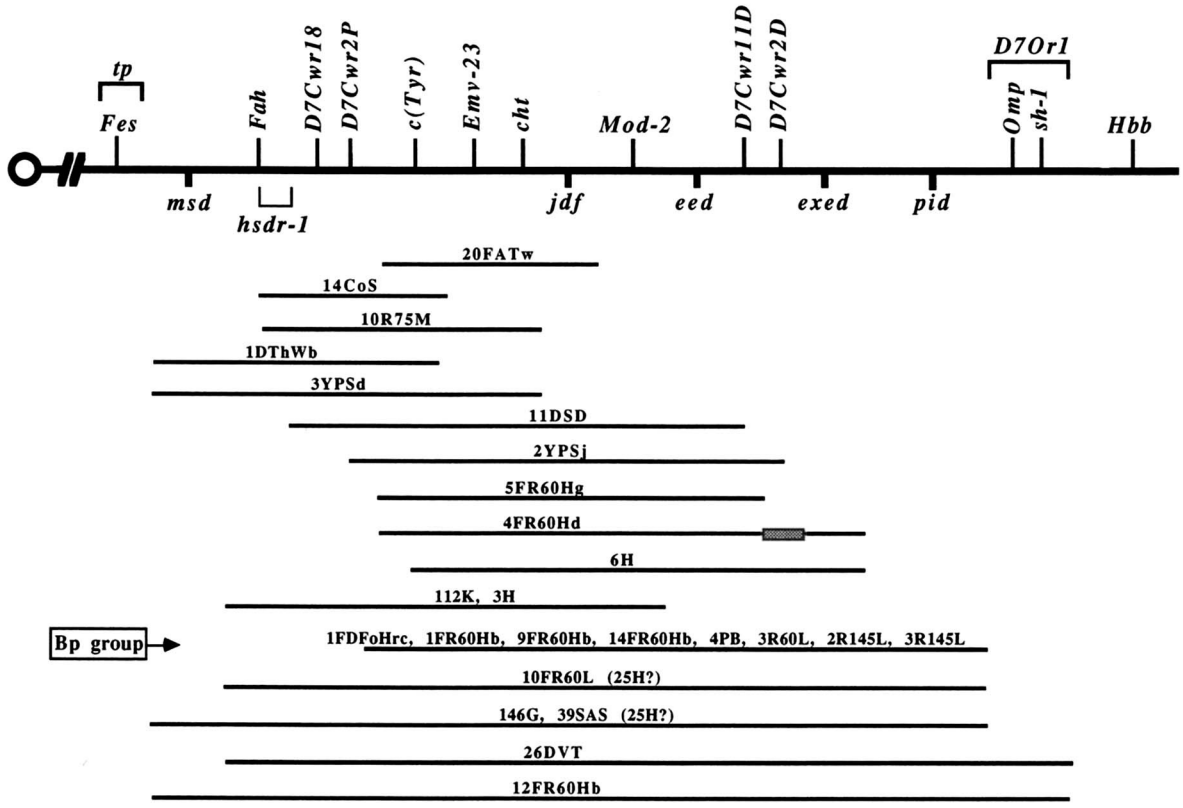

FIGURE 1.—The *Fes–Hbb* region of mouse chromosome 7, incorporating the hypothesized extents of a number of lethal albino (*c*)-locus deletions. Loci on the genetic map are indicated in italics above the chromosome and include: *Fes*, feline sarcoma oncogene; *tp*, taupe; *Fah*, fumarylacetoacetate hydrolase; *c* (*Tyr*), albino (tyrosinase); *Emv-23*, integration site of ecotropic murine leukemia virus-23; *cht*, chocolate; *Mod-2*, mitochrondrial malic enzyme; *Omp*, olfactory marker protein; *sh-1*, shaker-1; *Hbb*, globin, β-chain; and the anonymous loci *D7Cwr18*, *D7Cwr2P*, *D7Cwr11D* and *D7Cwr2D*, and *D7Or1*. The centromere is indicated by the circle at the left of the map. Functional units, defined by complementation analyses of lethal albino (*c*)-locus mutations, are indicated directly below the chromosome and include: *msd*, mesoderm deficient; *hsdr-1*, hepatocyte-specific developmental regulation-1; *jdf*, juvenile development and male fertility; *eed* and *exed*, development of embryonic and extraembryonic ectoderm, respectively; *pid*, preimplantation development. See RINCHIK *et al.* (1992) for locus and mapping references. Horizontal lines below the map indicate the extent of deletions with respect to the functional map, and no correlation with a physical distance is implied. The designation for each deletion is indicated directly above each line, and the occurrence of more than one designation above a line indicates that those deletions cannot be discriminated from each other by either genetic or molecular criteria. The eight deletions of the Bp group are indicated. The shaded box over a segment of the $c^{4FR60Hd}$ deletion denotes the region "skipped" by this deletion (*i.e.*, mice homozygous for this deletion exhibit the *exed* phenotype, but the deletion does not remove the *D7Cwr2D* locus) (SHARAN *et al.* 1991). An uncertainty about which complementation group contains the c^{25H} deletion is indicated by the question marks. *Fes* maps 1.2–9 cM proximal to *c* (SAUNDERS and SELDIN 1990), *Omp* maps proximal to *sh-1* (BROWN *et al.* 1992), the anonymous locus *D7Or1* maps to the indicated deletion interval around the *sh-1* locus (JOHNSON, HAND and RINCHIK 1989), and *cht* maps to the *Emv-23–jdf* interval (POTTER and RINCHIK 1993).

carrying deletion breakpoints of c^{11DSD} and c^{2YPSj} have been cloned (SHARAN *et al.* 1991). Cloning of these breakpoints thus provided initial molecular-access points to the genetic regions required for the development of embryonic and extraembryonic ectoderm (loci *eed* and *exed* in Figure 1).

Embryos homozygous for any of the eight deletions of the Bp complementation group (RUSSELL, MONTGOMERY and RAYMER 1982) (see Figure 1) fail to elicit a decidual response and, thus, are thought to die before implantation (RUSSELL and RAYMER 1979). The preimplantation-lethal phenotype associated with homozygosity for the Bp deletions has not yet been analyzed to the same degree as has the c^{25H} deletion (LEWIS 1978). Complementation analyses suggest that the proximal breakpoints of these deletions lie between *hsdr-1* and *c* (*Tyr*) (or, perhaps, within *c*), and the distal breakpoints map distal to (or within) a locus (or loci) required for normal preimplantation development [the *pid* (*p*reimplantation *d*evelopment) locus; formerly designated "PS"] (RUSSELL, MONTGOMERY and RAYMER 1982). On the basis of genetic evidence and the phenotype of gross time-of-death, the eight Bp deletions and the c^{25H} deletion were all hypothesized to affect the *pid* function (RUSSELL, MONTGOMERY and RAYMER 1982).

Although the lethal phenotype of embryos homozygous for c^{25H} has been detailed, the number or the nature of genes mapping to the *pid* region remain unknown. It is likewise not yet known whether homozygosity for each of the mutations affecting *pid* leads to the same lethal phenotype. Thus, molecular

characterization of the *pid* deletions themselves, as well as obtaining molecular access to the *pid* region, are necessary first steps for ascertaining the role played by the *pid* region in preimplantation development. This report describes the molecular mapping of the proximal breakpoints of the Bp deletions with clones derived from the *Fah–Tyr* interval, the cloning of a restriction fragment carrying the breakpoint of one of the deletions ($c^{9FR60Hb}$) and the subsequent derivation a DNA probe mapping either within, or immediately distal to, the *pid* region.

MATERIALS AND METHODS

Mice: All mice were maintained at the Biology Division of Oak Ridge National Laboratory. Lethal albino mutations (c^l) were detected in heterozygosis with the c^{ch} (chinchilla) allele of the albino locus in the F_1 progeny of irradiated or control (101 × C3Hf)F_1 males or females (RUSSELL, RUSSELL and KELLY 1979). Homozygous-lethal albino mutations are maintained in the heterozygous state opposite the c^{ch} mutation by crosses of heterozygotes (c^{ch}/c^l) to a closed-colony, non-inbred c^{ch}/c^{ch} stock (2A). The heterozygotes are a light-chinchilla color, in contrast to the full, darker, chinchilla (c^{ch}/c^{ch}) phenotype.

Fetuses that were compound heterozygotes for c^ls that complement for early prenatal lethality (*e.g.*, $c^{l\text{-}1}$ and $c^{l\text{-}2}$) were produced from crosses of $c^{ch}/c^{l\text{-}1}$ × $c^{ch}/c^{l\text{-}2}$ and were identified as those fetuses lacking eye pigment at day 14.5 (E14.5) of gestation. The day of finding a vaginal plug was considered to be E0.5. Breeding protocols used for deriving animals carrying lethal *c* deletions opposite *Mus spretus* chromosomes 7 have been described elsewhere (JOHNSON, HAND and RINCHIK 1989).

Genomic DNAs and hybridization protocols: The methods for preparation of high-molecular-weight DNA from mouse spleens or from whole 14.5-day fetuses, Southern blotting and filter hybridizations have been described elsewhere (RINCHIK *et al.* 1990).

Hybridization probes: MTY811, a 1.4-kb *Eco*RI fragment of mouse tyrosinase cDNA (KWON *et al.* 1988), was used as a probe for tyrosinase-exon-containing restriction fragments in genomic DNA. MTY811 is not a full-length cDNA, but it detects the same exon-containing fragments as does the full-length cDNA (B. S. KWON, unpublished data). A 0.9-kb *Eco*RI-*Acc*I fragment, designated N9.9, was used as a probe for the *Pmv-31* integration site (RINCHIK *et al.* 1993). E305 and E336B, identifying the loci *D7Rt305* and *D7Rt336*, respectively, are 4.0-kb and 0.4-kb *Eco*RI fragments derived from microdissected Rb(7.18)9Lub chromosomes and map within the c^{14CoS} deletion (TÖNJES *et al.* 1991). The *D7Cwr18* locus was detected by pTM.E2a (KELSEY *et al.* 1992). A chromosome-9 probe, 94.1, a 0.5-kb *Eco*RI-*Hin*dIII fragment (RINCHIK *et al.* 1986), was used as a positive hybridization control in experiments involving mice carrying homozygous or compound-heterozygous deletions of chromosome 7.

Cloning of a restriction fragment carrying the $c^{9FR60Hb}$ deletion breakpoint: *Bam*HI-digested spleen DNA from $c^{ch}/c^{9FR60Hb}$ mice was size fractionated by sucrose gradient centrifugation by standard procedures (SAMBROOK, FRITSCH and MANIATIS 1989). Fractions containing the 3.6-kb fragment, identified by Southern blot analysis using E336B as a hybridization probe, were pooled, ligated to *Eco*RI/*Bam*HI-digested λ2001 (American Type Culture Collection) and packaged *in vitro* (Gigapack Gold II, Stratagene). Approximately 6.5 × 10^5 recombinant phage, presumably containing several co-ligated *Bam*HI fragments (λ2001 accepts inserts of 9–23 kb), were plated on the selective host, *Escherichia coli* NM539. Plaque lifts were screened with the E336B clone, yielding four positive phage, and one of these was purified. *Bam*HI digestion of DNA from this positive phage yielded fragments of 6.8, 4.5, 3.6, 3.4 and 2.0 kb in size; the 3.6-kb fragment was found to hybridize to the E336B probe, and it was subcloned into *Bam*HI-digested pBluescript KS plasmid vector (Stratagene) to yield the clone pRN303. An ~400-bp *Rsa*I fragment of this clone, designated RN303.1, which contained sequence from the distal end of the $c^{9FR60Hb}$ deletion as well as some vector sequence, was used for the mapping analyses described in RESULTS.

RESULTS

Analysis of preimplantation-lethal albino mutations (c^l) of the Bp complementation group with a tyrosinase cDNA clone: One strategy for gaining molecular entry to the *pid* locus in chromosome 7 is to identify structural rearrangements associated with the proximal breakpoints of albino deletions that are lethal during preimplantation stages of development when homozygous (*e.g.*, the eight deletions of the Bp group; see Figure 1). Cloning of rearranged restriction fragments that contain the site of any Bp deletion breakpoint would then provide cloned DNA sequences from the distal end of the deletion, which would map immediately distal to or within the *pid* locus. Previous complementation analyses (RUSSELL, MONTGOMERY and RAYMER 1982) suggested that the proximal breakpoints of the eight Bp deletions mapped between *hsdr-1* and *c* (*Tyr*; tyrosinase) or, perhaps, within *Tyr* itself. Thus, it was of interest to map the proximal extents of the Bp deletions with respect to DNA-defined loci mapping to the *Fah–Tyr* interval. This analysis would also include a determination of whether any Bp deletion disrupted the 70 kb of DNA associated with the tyrosinase transcription unit itself (RUPPERT *et al.* 1988).

It has been reported previously (KWON *et al.* 1987; RUPPERT *et al.* 1988) that both human and mouse *Tyr* probes fail to hybridize to DNA derived from animals homozygous for the c^{3H} deletion. The c^{3H} deletion (GLUECKSOHN-WAELSCH 1979) was provisionally assigned to the "E" complementation group by RUSSELL, MONTGOMERY and RAYMER (1982) on the basis of its similarity to the c^{112K} deletion (see Figure 1). We found that, as expected, the MTY811 tyrosinase cDNA probe fails to hybridize to DNA derived from fetuses homozygous for c^{112K} (Figure 2A). Consequently, each of the Bp mutations could be tested for deletion of *Tyr* coding sequences by Southern-blot analysis of DNA from individuals that carried such a mutation (c^{Bp}) in compound heterozygosis with the c^{112K} mutation. Therefore, DNAs derived from E14.5 c^{Bp}/c^{112K} fetuses were digested with restriction enzymes, blotted to nylon filters and hybridized with the tyrosinase

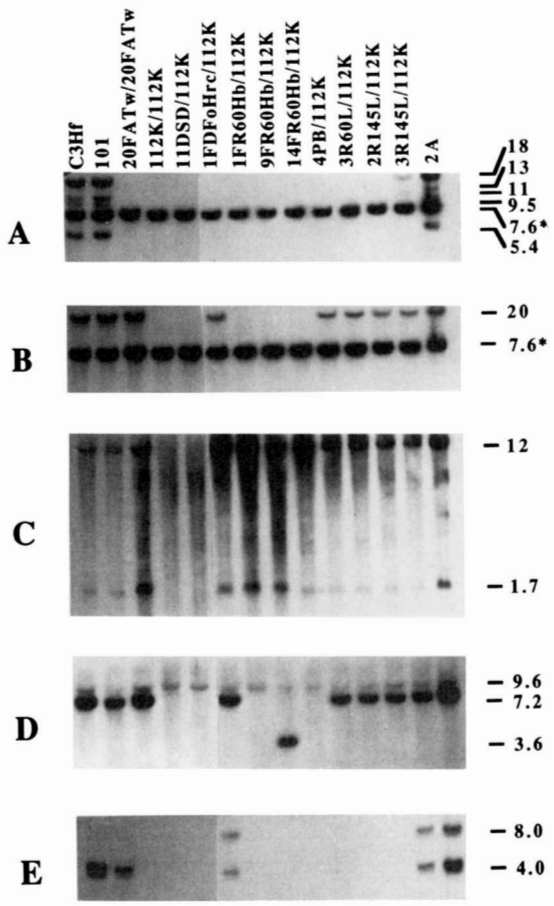

FIGURE 2.—Southern blot analysis of lethal albino mutations with probes from the *Fah–Tyr* interval. Splenic or fetal DNAs from individuals of the indicated genetic constructions were digested with *Bst*EII (A, B), *Eco*RI (C, E) or *Bam*HI (D), electrophoresed in agarose, blotted to nylon and hybridized with the following radiolabeled probes: (A) tyrosinase cDNA clone MTY811 (*Tyr*); (B) N9.9 (*Pmv-31*); (C) pTM.E2a (*D7Cwr18*); (D) E336B (*D7Rt336*); (E) E305 (*D7Rt305*). Fragment sizes are given in kilobases. The 7.6-kb fragment marked with an asterisk in panels A and B results from hybridization to the chromosome-*9* control probe p94.1. The blots in panels C, D and E have been hybridized to control probes demonstrating that there is hybridizable DNA in each lane (data not shown). E305 (panel E) hybridizes very weakly to a 0.6-kb *Eco*RI fragment, which is present each time the 4-kb fragment is present; however, this area of the gel is not shown in this figure.

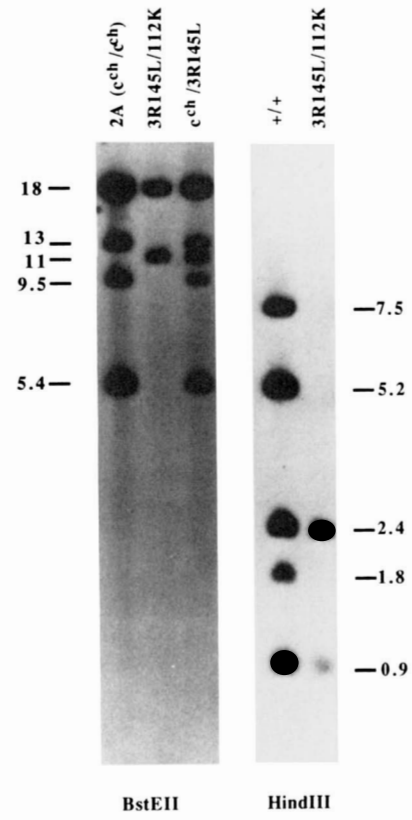

FIGURE 3.—Southern blot analysis of the c^{3R145L} mutation with a tyrosinase cDNA probe. DNAs from individuals of the indicated genetic constructions were digested with the indicated restriction enzyme, electrophoresed in agarose, blotted to nylon and hybridized with the tyrosinase cDNA clone MTY811. Fragment sizes are given in kilobases.

cDNA probe MTY811. Figure 2A shows a representative blot in which MTY811 recognizes four *Bst*EII fragments (18, 13, 9.5 and 5.4 kb in size) in C3Hf/Rl (+/+), 101/Rl (+/+), and 2A (c^{ch}/c^{ch}) DNA. With the exception of the c^{3R145L}/c^{112K} compound heterozygote, each lethal *c* mutation of the Bp group is completely deleted for MTY811 sequences; only the 7.6-kb *Bst*EII fragment associated with the chromosome-*9* control probe p94.1 is evident.

Sequences capable of hybridizing to MTY811 were detected in DNA from the c^{3R145L}/c^{112K} compound heterozygote. Figure 2A and Figure 3 show that c^{3R145L}/c^{112K} DNA exhibits a wild-type 18-kb *Bst*EII fragment, is deleted for the remaining three wild-type fragments and manifests a novel 11-kb fragment. As expected, five *Bst*EII fragments (four wild type plus the novel 11-kb) can be detected in heterozygous c^{ch}/c^{3R145L} DNA (Figure 3). These results suggest that the proximal c^{3R145L} deletion breakpoint lies somewhere within the 70 kb of genomic DNA spanned by the *Tyr* transcription unit, and that the 11-kb *Bst*EII fragment could possibly represent a deletion breakpoint-fusion fragment carrying, both, tyrosinase sequences and sequences derived from the distal end of the albino deletion complex (between *pid* and *Omp* on the map in Figure 1).

Because the genomic order of the four wild-type *Bst*EII fragments capable of hybridizing to the tyrosinase cDNA probe is unknown, a more precise localization of the c^{3R145L} breakpoint within the tyrosinase gene was accomplished by hybridizing MTY811 to a Southern blot of *Hin*dIII-digested DNA from a c^{3R145L}/c^{112K} compound heterozygote. RUPPERT *et al.* (1988) had demonstrated that the mouse tyrosinase cDNA clone recognizes five *Hin*dIII fragments, which could be ordered as follows: 5′–5.2 kb–7.5 kb–1.8 kb—0.9 kb–2.4 kb–3′. Figure 3 demonstrates that the c^{3R145L} deletion removes the 5.2-, 7.5- and 1.8-kb *Hin*dIII fragments, *i.e.*, the three most-5′ exon-containing fragments. Consequently, it follows that the

tyrosinase gene is oriented with its 3′ end toward the centromere since the distally extending c^{3R145L} deletion removes the 5′ end.

Deletion mapping of loci within the *Fah–Tyr* interval: The proximal extents of the remaining seven Bp deletions were mapped with respect to other loci defined by DNA probes that map to the *Fah–Tyr* interval. Figure 2B shows the results of hybridizing N9.9, a probe from the *Pmv-31* locus (RINCHIK *et al.* 1993), to the blot of c^{Bp}/c^{112K} and other c^{l}/c^{112K} DNAs depicted in Figure 2A. The N9.9 probe recognizes a 20-kb *Bst*EII fragment in wild-type and c^{ch}/c^{ch} DNA. It fails to hybridize to DNA from c^{112K}/c^{112K} homozygotes and hybridizes only to DNAs derived from c^{20FATw}/c^{20FATw} homozygotes and $c^{1FDFoHrc}/c^{112K}$, c^{4PB}/c^{112K}, c^{3R60L}/c^{112K}, c^{2R145L}/c^{112K} and c^{3R145L}/c^{112K} compound heterozygotes.

The loci *D7Cwr18*, *D7Rt336* and *D7Rt305*, defined by anonymous clones pTM.E2a, E336B and E305, respectively, all map within the c^{14CoS} deletion, but proximal to *Tyr* (NISWANDER *et al.* 1991; TÖNJES *et al.* 1991; KELSEY *et al.* 1992). Therefore, these probes were also tested for their ability to hybridize to homozygous or compound heterozygous DNAs derived from animals carrying lethal deletions of the Bp group. As expected, each probe fails to hybridize to DNA from c^{112K}/c^{112K} homozygotes; therefore, each of the lethal albino mutations could be directly tested for deletion of these particular loci by examining the c^{Bp}/c^{112K} DNAs as described above. Hybridization of the pTM.E2A probe to *Eco*RI-digested DNAs (Figure 2C) shows that wild-type 12- and 1.7-kb *Eco*RI fragments are detected in DNA from all genotypes except c^{112K}/c^{112K} and c^{11DSD}/c^{112K}. Thus, *D7Cwr18* is deleted in none of the Bp mutations, being deleted only in the neonatally lethal c^{112K} deletion and in the postimplantation lethal deletion c^{11DSD} as reported previously (NISWANDER *et al.* 1991).

Hybridization of the E336B probe (*D7Rt336*) to *Bam*HI-digested DNAs (Figure 2D) detects a 7.2-kb *Bam*HI fragment in wild-type DNA and in DNAs derived from c^{20FATw}/c^{20FATw}, $c^{1FDFoHrc}/c^{112K}$, c^{4PB}/c^{112K}, c^{3R60L}/c^{112K}, c^{2R145L}/c^{112K}, and c^{3R145L}/c^{112K} mice. The probe fails to hybridize to c^{112K}/c^{112K}, c^{11DSD}/c^{112K}, $c^{1FR60Hb}/c^{112K}$ and $c^{14FR60Hb}/c^{112K}$. A minor, cross-hybridizing band of ~9.6 kb in size is found in all DNAs. Importantly, the E336B probe detects a size-altered 3.6-kb *Bam*HI fragment and no wild-type fragment in $c^{9FR60Hb}/c^{112K}$ DNA (see below).

Hybridization of the E305 probe (*D7Rt305*) to blots of *Eco*RI-digested DNAs (Figure 2E) showed that, with the exception of $c^{1FDFoHrc}$ and c^{3R145L}, the wild-type 4-kb fragment at the *D7Rt305* locus was deleted in all Bp-group deletions. Interestingly, this probe detects an additional fragment in 2A-c^{ch}/c^{ch} DNA, approximately 8 kb in size, and both $c^{1FDFoHrc}$ and c^{3R145L} carry the extra 2A fragment even though they were induced on either a C3Hf or 101 chromosome, neither of which carries the 8-kb fragment. Because these two deletions have been maintained by crosses to 2A for many years, it is likely that a crossover occurred between *D7Rt305* and the proximal deletion breakpoint in each case, thereby placing the 2A polymorphism on the mutant chromosome.

Cloning of a genomic rearrangement at the *D7Rt336* locus in the $c^{9FR60Hb}$ mutation: To determine whether the size-altered 3.6-kb *Bam*HI fragment detected at the *D7Rt336* locus in $c^{9FR60Hb}/c^{112K}$ DNA (Figure 2D) was indicative of a rearrangement caused by the proximal breakpoint of the $c^{9FR60Hb}$ deletion, c^{ch}/c^{ch}, +/+ and $c^{9FR60Hb}/c^{112K}$ DNAs were digested with a variety of restriction enzymes, and the resultant blots were hybridized with E336B. Size-altered fragments were also found in $c^{9FR60Hb}/c^{112K}$ DNA following digestion with *Hin*dIII, *Pst*I, *Pvu*II, *Xba*I, *Acc*I and *Kpn*I (data not shown). These results suggested that the 3.6-kb *Bam*HI fragment detected by E336B in $c^{9FR60Hb}$ DNA is due to a genomic rearrangement rather than the simple gain of a *Bam*HI restriction site.

To derive a probe for DNA mapping at the distal end of the $c^{9FR60Hb}$ deletion, the 3.6-kb fragment from size-selected, *Bam*HI-digested $c^{ch}/c^{9FR60Hb}$ spleen DNA was cloned in a lambda vector (see MATERIALS AND METHODS). The fragment RN303.1, which contains sequences derived from one end of the 3.6-kb fragment as well as some vector sequence (Figure 4A), detects *Eco*RI restriction-fragment-length variants (RFLVs) between laboratory mouse and *M. spretus* DNA (a prominent 0.6-kb fragment, along with a cross-hybridizing ~10-kb fragment and a weakly cross-hybridizing 5.4-kb fragment in C3Hf/Rl and 101Rl DNA; a 5.2-kb fragment in 2A-c^{ch}/c^{ch} DNA; and a 5.7-kb fragment in *M. spretus* DNA). To deletion-map the primary locus defined by this probe, blots of *Eco*RI-digested DNAs derived from c^{ch}/c^{l} carriers and from F_1 progeny carrying each c^{l} opposite a *M. spretus* chromosome 7 (JOHNSON, HAND and RINCHIK 1989) of all deletions thought to affect *pid* ($c^{1FDFoHrc}$, $c^{1FR60Hb}$, $c^{9FR60Hb}$, $c^{14FR60Hb}$, c^{4PB}, c^{3R60L}, c^{2R145L}, c^{3R145L}, $c^{10FR60L}$, c^{146G}, c^{39SAS}) (see Figure 1), along with presumed deletion and nondeletion controls, were hybridized with RN303.1. Figure 4B demonstrates that RN303.1 detects the 5.7-kb *M. spretus* fragment, but not the 0.6-kb C3H/101 fragment, in F_1 mice carrying *M. spretus* chromosomes 7 and the deletions $c^{1FDFoHrc}$, $c^{14FR60Hb}$, c^{4PB}, c^{3R60L}, $c^{10FR60L}$, c^{146G}, c^{39SAS}, c^{26DVT} or $c^{12FR60Hb}$; however, the probe does detect the 0.6-kb C3H/101 fragment in F_1s carrying $c^{1FR60Hb}$, $c^{9FR60Hb}$, c^{2R145L} and c^{3R145L} in addition to deletions (c^{3YPSd}, c^{11DSD}, $c^{4FR60Hd}$, $c^{5FR60Hg}$ and c^{2YPSj}) not expected to include the site of the $c^{9FR60Hb}$ distal breakpoint. The 5.2-kb 2A-specific

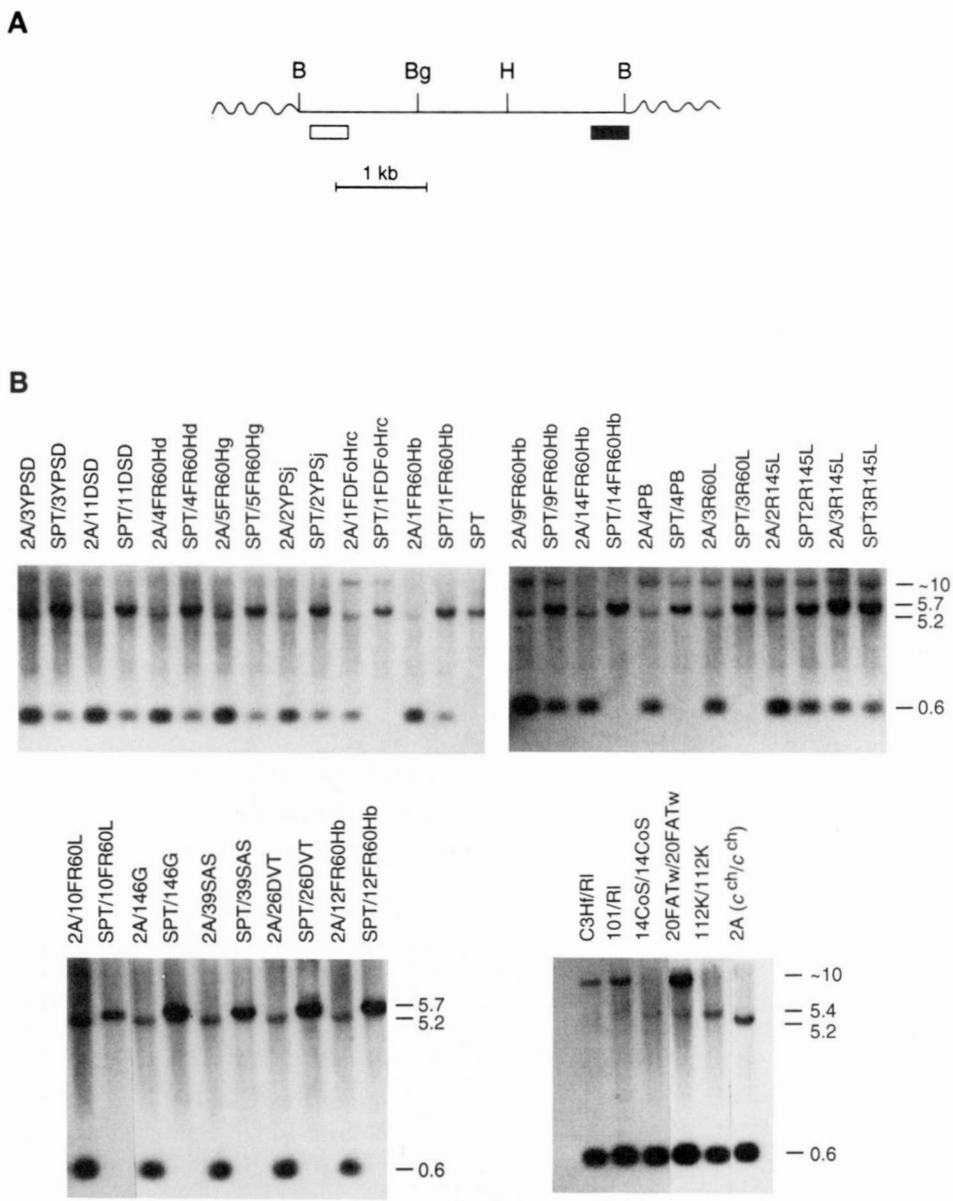

FIGURE 4.—Deletion mapping of the *D7Rn6* locus. (A) A restriction map of RN303, a 3.6-kb *Bam*HI fragment containing DNA sequences from both sides of the $c^{9FR60Hb}$ deletion. B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII. The E336B probe (open box), a 445-bp genomic *Eco*RI fragment defining the *D7Rt336* locus (TÖNJES *et al.* 1991), hybridizes to the 1.3-kb *Bam*HI–*Bgl*II fragment; the RN303.1 probe (filled box), corresponding to the *D7Rn6* locus, hybridizes to the 1.3-kb *Bam*HI–*Hin*dIII fragment. Vector sequences (from pBluescript) are indicated by the wavy lines. The RN303.1 probe contains approximately 290 bp of mouse DNA plus a small amount of vector sequence. Additional restriction mapping data (not shown) provide evidence for at least three *Eco*RI sites within the 1.3-kb *Bam*HI–*Bgl*II fragment, but the orientation of these sites within the fragment, and, consequently, the exact location of the E336B probe, were not determined. (B) Splenic DNAs from individuals of the indicated genetic constructions (or fetal DNAs from homozygotes) were digested with *Eco*RI, electrophoresed in agarose, blotted to nylon filters, and hybridized with RN303.1. Fragment sizes are given in kilobases. 2A = a chromosome 7 marked with c^{ch}, derived from the 2A stock; SPT = *M. spretus* chromosome 7. The indicated genotypes are shortened versions of the true genotype; *e.g.*, "2A/3YPSd" = c^{ch}/c^{3YPSd}; "SPT/3YPSd" = $+^{SPT}/c^{3YPSd}$; and "SPT" = $+^{SPT}/+^{SPT}$. The presence or absence of the 0.6-kb C3Hf or 101 *Eco*RI fragment was used to map the *D7Rn6* locus; the variably cross-hybridizing ~10-kb and 5.4-kb *Eco*RI fragments that were not analyzed further (see text) are also shown.

fragment is never observed in the *M. spretus* F_1s because these do not carry 2A DNA closely linked to *c* (JOHNSON, HAND and RINCHIK 1989). Thus, the deletion profile for *D7Rn6*, the locus defined by the segregation pattern of the 0.6-kb *Eco*RI fragment, is consistent with its placement between *exed* and *sh-1*, either distal to or within *pid*. [The RN303.1 probe also detects a cross-hybridizing *Eco*RI fragment of ~10 kb in size in several parental and F_1 DNAs in this panel. The presence of this fragment could not be correlated with partial digestion, and its map position could not be precisely determined; at present, we cannot rule out a locus of C3Hf or 101 origin linked to *c*, but not within the deletions, that has been carried along with the deletion during maintenance crosses of the mutant chromosome to stock 2A (which does not have the fragment). The very weakly and inconsistently hybridizing 5.4-kb fragment sometimes observed in C3Hf or 101 DNA was not analyzed further.]

Figure 4B also shows that the 0.6-kb fragment associated with *D7Rn6* is not deleted in DNA derived from mice homozygous for the c^{14CoS}, c^{112K} and c^{20FATw} deletions. Additional confirmation of the linkage of *D7Rn6* to the *c* (*Tyr*)–*Hbb* interval was obtained by hybridizing RN303.1 to Southern blots of *Eco*RI-digested DNAs obtained from segregants of the interspecific backcross (129/Rl-*p* c^{ch}/p c^{ch} × *M. spretus*)F_1 × 129/Rl-*p* c^{ch}/p c^{ch} (JOHNSON, HAND and RINCHIK 1989). No recombination between the 5.7-kb *M. spre-*

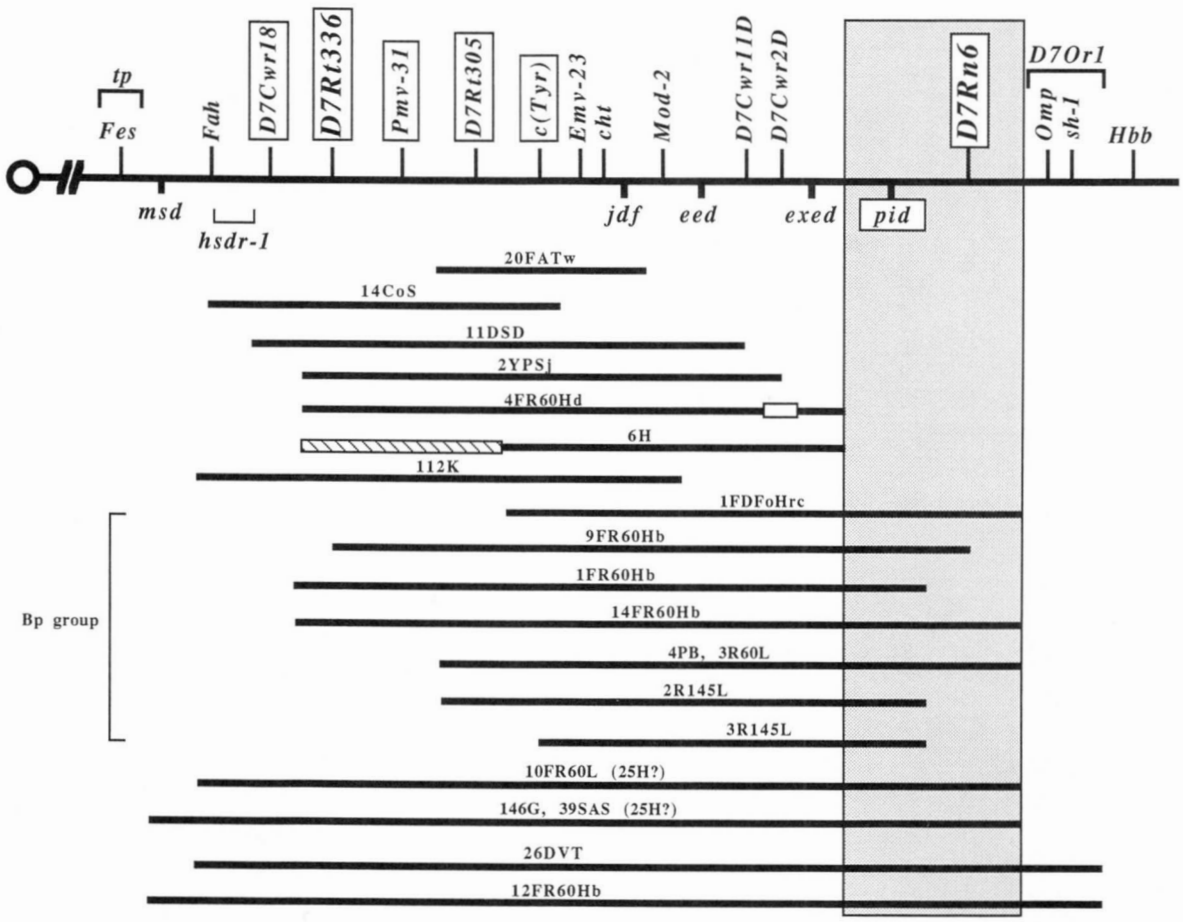

FIGURE 5.—A refined deletion map of the *Fes–Hbb* interval including the location of breakpoints mapping near the preimplantation-development (*pid*) locus. The map from Figure 1 has been modified to incorporate the data from Figures 2 and 4. The breakpoints of the $c^{9FR60Hb}$ deletion (*D7Rt336* and *D7Rn6*) are shown in larger type. The proximal breakpoint of the c^{3R145L} deletion is shown to be within the *Tyr* transcription unit (see Figure 3). The large shaded box highlights the region around the *pid* locus that is now further subdivided by the distal breakpoints of some of the Bp deletions. Loci above the chromosome appearing within a box represent those loci mapped by the data in Figures 2 and 4. Not all of the deletions analyzed in Figure 4 are included. The *D7Cwr2P* locus was not mapped on the Bp deletions and, therefore, is not included in this map. The c^{6H} deletion was not analyzed with respect to the loci analyzed in this study; therefore, the diagonally striped box denotes an uncertainty about its proximal extent. The open box denotes the region skipped by the $c^{4FR60Hd}$ deletion (see Figure 1 legend).

tus RFLV and *M. spretus*-derived RFLVs for *c* (*Tyr*) and *Hbb* was observed in 49 segregants (data not shown). A refined map of the *Fes–Hbb* region, based on the data presented in Figures 2 and 4B, is presented in Figure 5.

DISCUSSION

The numerous *c*-locus mutations are being exploited as genetic reagents for developing physical maps, as well as more detailed fine-structure functional (mutation) maps, of the *Fes–Hbb* region of chromosome 7 (JOHNSON, HAND and RINCHIK 1989; RINCHIK and RUSSELL 1990; RINCHIK, CARPENTER and SELBY 1990; NISWANDER *et al.* 1989, 1991; SHARAN *et al.* 1991; KELSEY *et al.* 1992). This report describes the fine-structure mapping of a number of DNA-defined loci into intervals defined by the proximal breakpoints of a number of deletions that are homozygous-lethal during preimplantation stages of embryogenesis. Such fine-structure deletion mapping of DNA-defined loci provides one framework for the initiation, orientation and closure of physical maps of the region associated with the albino deletions. This type of physical mapping, together with the analysis of the mutant phenotypes specified by the deletions themselves and with the use of the deletions for the fine-structure genetic mapping of a number of *N*-ethyl-*N*-nitrosourea-induced presumed point mutations (see accompanying report, RINCHIK, CARPENTER and LONG 1993), comprise the central components of one strategy for defining the molecular and functional complexity of the *Fes–Hbb* region and for elucidating the roles played by genes of this region in normal mammalian development.

If one assumes that the Bp deletions are simple and linear, then the results presented here indicate the

genetic order within the *Fah–Tyr* interval to be: *D7Cwr18–D7Rt336–Pmv-31–D7Rt305*. We have data (M. L. KLEBIG and E. M. RINCHIK, unpublished data) that neither *Pmv-31* nor the more proximal locus *D7Rn2* (from the *Fah* gene) (KLEBIG, RUSSELL and RINCHIK 1992) recombines with *Tyr* in 182 segregants of an interspecific backcross described in a previous report (JOHNSON, HAND and RINCHIK 1989). By using the independent *c*-locus deletions, it has now been possible to order these very closely linked clones that would otherwise segregate as a completely linked block in an interspecific backcross. The *Fah–Tyr* interval has recently been mapped physically (KELSEY *et al.* 1992), allowing this deletion map to be accurately correlated with the physical map. If it becomes important in the future to identify and clone rearrangements associated with any of the Bp-group deletions for the purpose of jumping into regions distal to *D7Rn6* (*e.g.*, see RINCHIK, CARPENTER and LONG 1993, accompanying report), the data presented here, along with knowledge of the physical map, will facilitate such cloning exercises.

Seven of the eight of the Bp mutations are completely deleted for tyrosinase coding sequences. The one exception is the c^{3R145L} mutation, which is partially deleted for a portion of the tyrosinase gene and manifests a size-altered *Bst*EII restriction fragment that hybridizes to tyrosinase coding sequences. Southern-blot analysis of *Hin*dIII digests of c^{3R145L}/c^{112K} DNA demonstrated that the three most-5′ exon-containing fragments have been deleted in this mutation. Because the c^{3R145L} deletion extends distally from *c* [it is deleted for *Emv-23* (E. M. Rinchik, unpublished data) and *Mod-2* (RUSSELL, MONTGOMERY and RAYMER 1982)], the tyrosinase gene must be oriented with its 5′ end closer to the telomere and its 3′ end closer to the centromere.

The *pid* locus is currently defined by the failure of embryos homozygous either for one of the eight Bp deletions or for $c^{10FR60L}$, c^{146G}, c^{39SAS}, c^{26DVT} and $c^{12FR60Hb}$ to elicit a uterine decidual reaction (RUSSELL and RAYMER 1979; RUSSELL, MONTGOMERY and RAYMER 1982). Homozygosity for the c^{25H} deletion (LEWIS 1978), which was placed into the same complementation group as either the $c^{10FR60L}$ deletion or the c^{146G} and c^{39SAS} deletions (Figure 5) on the basis of published genetic evidence (RUSSELL, MONTGOMERY and RAYMER 1982), is associated with an arrest of cleavage at the three- to six-cell stage and an apparent abnormality in mitosis or cytokinesis (LEWIS 1978). More detailed phenotypic analyses of early cleavage embryos from crosses that can generate Bp-deletion homozygotes may indicate whether *pid* could be a single gene (if, for example, the same phenotype is observed in embryos homozygous for each of the deletions affecting *pid*) or whether the *pid* phenotype may be more complex, being the result of a disruption of two or more genes that have distinct functions essential for progressing through preimplantation development. Still another alternative is that because the *pid* deletions are relatively large, the *pid* phenotype may be the result of the combinatorial deletion of a number of genes not necessarily required during preimplantation stages. However, the rather specific nature of the c^{25H} lethal phenotype (LEWIS 1978) raises questions about whether any combinatorial deletion of loci normally acting after implantation has any bearing on the preimplantation lethality of these deletions.

The isolation of a distally mapping subclone of a size-altered 3.6-kb *Bam*HI fragment, detected at the *D7Rt336* locus in the $c^{9FR60Hb}$ chromosome, has defined the *D7Rn6* locus and has provided access to the *pid* region. A new physical map can now be nucleated from *D7Rn6*, and its proximal-distal orientation can easily be determined with respect to other deletion breakpoints that map to this interval (see Figure 5). The mapping of the distal breakpoint of the $c^{9FR60Hb}$ deletion (*i.e.*, the *D7Rn6* locus) suggests that the $c^{1FR60Hb}$, c^{2R145L} and c^{3R145L} deletions do not extend as far distally as do other deletions affecting *pid*; they must, however, break either within or distal to *pid*. In combination, these facts suggest that these three deletions would be more suitable than the other (longer) deletions affecting *pid* for defining the extent of *pid* on any physical map. In this context, it is important to note that the more distal of the two Bex-group deletion breakpoints (c^{6H} and $c^{4FR60Hd}$; NISWANDER *et al.* 1989, 1991; SHARAN *et al.* 1991) defines the proximal boundary of the *pid* locus. Consequently, it will be important to locate the c^{6H} or $c^{4FR60Hd}$ breakpoint on any physical map extended proximally from *D7Rn6*, as this would provide the necessary stopping point in the search for the *pid* gene(s). Likewise, it will be interesting to determine whether embryos homozygous for the (distally) shorter $c^{1FR60Hb}$, c^{2R145L} or c^{3R145L} deletions have the same phenotype as embryos homozygous for deletions that remove *D7Rn6*. Perhaps this type of experiment would provide some data on whether the preimplantation lethality of these mutations is caused by the absence of a single gene or of more than one gene.

In parallel with construction of a physical map of the *pid* region, larger stretches of DNA around *D7Rn6*, cloned into either cosmids or yeast artificial chromosomes (YACs), can be analyzed for the presence of coding sequences by a variety of methods such as exon-amplification (BUCKLER *et al.* 1991), the DNA sequence-based Coding Recognition Module (UBERBACHER and MURAL 1991), direct selection of cDNA clones by hybridization to YAC clones (LOVETT, KERE and HINTON 1991; PARIMOO *et al.* 1991), or by more standard methods of identification and cloning of CpG

islands or evolutionarily conserved sequences. Coding sequences identified by any of these methods could then be analyzed further as possible candidates for the gene or genes contributing to the *pid* preimplantation-lethal phenotype.

In addition to the *pid* locus, which is defined by the lethal phenotype of homozygous *c* deletions, the *D7Rn6* locus also provides a needed point of molecular access to the proximal border of a region into which four new *N*-ethyl-*N*-nitrosourea (ENU)-induced, *post*implantation-lethal mutations have been found to map (RINCHIK, CARPENTER and LONG 1993, accompanying report). Thus, the development of physical maps nucleated on *D7Rn6*, combined with subsequent derivation of DNA clones mapping distally (from *D7Rn6* YACs, for example), should provide important initial steps in the identification of the loci defined by these new, presumably single-gene mutations mapping to the *pid–Hbb* interval of chromosome 7.

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